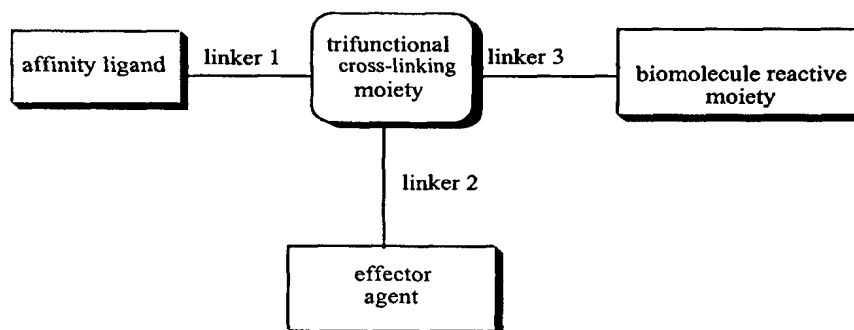




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(21) International Application Number: PCT/SE99/01241 (22) International Filing Date: 7 July 1999 (07.07.99) (30) Priority Data: PCT/SE98/01345 7 July 1998 (07.07.98) SE (71) Applicants (for all designated States except US): DEPT. OF RADIATION ONCOLOGY, UNIVERSITY OF WASHINGTON [US/US]; 2121 North 35th Street, Mailstop XD-48, Seattle, WA 98103 (US). MITRA MEDICAL TECHNOLOGY AB [SE/SE]; Ideon Research Park, S-223 70 Lund (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): WILBUR, D., Scott [US/US]; 6015 137th Place S.W., Edmonds, WA 98026 (US). SANDBERG, Bengt, E., B. [SE/SE]; Blomstergården 18, S-245 62 Hjärup (SE). (74) Agent: AWAPATENT AB; P.O. Box 5117, S-200 71 Malmö (SE).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: TRIFUNCTIONAL REAGENT FOR CONJUGATION TO A BIOMOLECULE



(I)

(57) Abstract

A reagent for conjugation to a biomolecule, wherein the reagent is a single molecule with at least three functional parts and has schematic structure (I): a) wherein a trifunctional cross-linking moiety is coupled to b) an affinity ligand via a linker 1, said affinity ligand being capable of binding with another molecule having affinity for said ligand, to c) an effector agent, optionally via a linker 2, said effector agent exerting its effect on cells, tissues and/or humours molecules *in vivo* or *ex vivo*, and to d) a biomolecule reactive moiety, optionally via a linker 3, said moiety being capable of forming a bond between the reagent and the biomolecule.

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TRIFUNCTIONAL REAGENT FOR CONJUGATION TO A BIOMOLECULE

Field of Invention

The present invention is directed to a reagent for the conjugation to a biomolecule for the diagnosis and treatment of human and animal conditions or diseases and
5 for the in vitro analysis of affinity labelled biomolecules.

More precisely, the present invention is generally directed at a novel chemical reagent which simultaneously conjugate an affinity ligand and an effector agent with a
10 biomolecule to obtain minimal modification of that biomolecule; to a method of diagnosis or treatment of a human or animal condition or disease; and to a kit comprising the reagent according to the present invention. As an example, chemical reagents which contain an
15 affinity ligand (e.g. a biotin moiety), an effector agent (e.g. a radiolabeling moiety), and a biomolecule reactive moiety are coupled together through a trifunctional cross-linking moiety and spaced apart with linker moieties. Using such a reagent, a biomolecule can be
20 biotinylated and radiolabeled via one of two methods, then employed in medical protocols, such as those utilizing extracorporeal immunoabsorptive removal methods to minimize the toxic effects to normal tissue and blood components.

Background of the Invention

Many biomolecules, including proteins and peptides, hold potential as reagents for use in diagnosis and therapy of human conditions and diseases. As most biomolecules do not, by themselves, have properties to make them
30 useful as diagnostic and/or therapeutic reagents, biomolecules of interest are often chemically modified to

achieve this. However, one very important criterion must be applied when chemically modifying biomolecules. That criterion is that the modification does not alter the biological property that is important (e.g. cancer cell targeting) in the use of that particular biomolecule. This criterion makes it imperative that site-selective (where possible) and minimal modification of the biomolecule be conducted.

Modification of a targeting biomolecule with an effector agent, such as a radionuclide, can provide valuable new tools for diagnosis and therapy of human and animal diseases or conditions. Coupling of a radionuclide to the biomolecule results in the desired diagnostic effect of providing photons that can be measured or imaged externally to show the localization of the radiolabeled biomolecule, or it may provide the desired therapeutic effect of causing damage to cells or tissues that are targeted by the biomolecule. Biomolecules labeled with photon emitting radionuclides can be used for the diagnosis of a number of human conditions (i.e. extent of myocardial infarcts, presence of cancer, etc.). For example, technetium-99m labeled antibodies can be used to diagnose cancer (Granowska et al. Eur. J. Nucl. Med. 20, 483-489, 1993; Lamki et al. Cancer Res. 50, 904s-908s, 1990; Goldenberg et al. Cancer Res. 50, 909s-921s, 1990); iodine-123 labeled fatty acids can be used to evaluate myocardial perfusion (Corbett J. Nucl. Med. 35, 32s-37s, 1994; Hansen J. Nucl. Med. 35, 38s-42s, 1994; Knapp et al. J. Nucl. Med. 36, 1022-1030, 1995); and fluorine-18 labeled fluorodeoxyglucose can be used to evaluate a variety of functions of the brain (Posner et al., Science 240, 1627-1631, 1988). Biomolecules labeled with particle emissions (e.g. beta, positron, alpha, Auger electrons)

can potentially be used for targeted radiotherapy of human disease such as cancer. For example, a large number of monoclonal antibodies (Behr et al. J. Nucl. Med. 38, 858-870, 1997; Divgi et al. J. Nucl. Med. 36, 586-592, 1995; DeNardo et al. Anticancer Res. 17, 1735-1744, 1997) and peptides (Zamora et al. Int. J. Cancer 65, 214-220, 1996; Stolz et al. Digestion 57, 17-21, 1996; Bender et al. Anticancer Res. 17, 1705-1712, 1997) labeled with therapeutic radionuclides such as iodine-131, yttrium-90 and Re-188 are being investigated as new reagents for cancer therapy. Thus, an important modification that can be carried out is to attach a functional moiety to the biomolecule which binds or bonds with a radionuclide. For small (i.e. < 2000 Da molecular weight) biomolecules, usually only one radionuclide binding/bonding moiety is site-selectively attached to cause minimal perturbation in its desired biological properties. Larger biomolecules, such as peptides and proteins, may be conjugated with more than one radionuclide binding/bonding moiety before loss of the desired biological properties, but these molecules generally retain more of their desired biological properties when minimal number of conjugations are obtained.

Modification of biomolecules with an "affinity ligand" is also important as it provides a means of coupling two entities together for a variety of *in vitro* and *in vivo* applications. By their nature, affinity ligands come in pairs. The preferred affinity ligands used for coupling to the biomolecule must have a high enough binding constant (e.g. 10^6 M^{-1} or greater) with a second compound to allow the two coupled entities to remain together for a period of time. An example of an affinity ligand pair is a monoclonal antibody and its hapten. The

affinity ligand pairs of biotin/avidin and biotin/streptavidin are often used with biomolecules. The very strong interaction (i.e. $K = 10^{13} - 10^{15} \text{ M}^{-1}$) of biotin with the proteins avidin and streptavidin (Green, Methods Enzymol. 184, 51-67, 1990; Green, Adv. Prot. Chem. 29, 85-133, 1975) provides a foundation for their use in a large number of applications, both for *in vitro* and *in vivo* uses. While the proteins avidin and streptavidin are sometimes conjugated with biomolecules, conjugation of biotin introduces less perturbation of the biomolecule, and more than one biotin molecule can be conjugated with minimal affect on the biomolecule. Therefore, the preferred affinity label is biotin or a derivative thereof, and the examples herein are reflective of this preference. As with the radionuclide binding/bonding moiety, it is important to minimize the number of affinity ligands (e.g. biotin conjugates) attached to a biomolecule to retain the desired biological properties.

Modification of the biomolecule by attachment (conjugation) of another molecule to a particular reactive functional group (e.g. amine, sulfhydryl, aldehyde, ketone) precludes attachment of a second molecule to that group. Thus, if attachment of more than one type of molecule to a biomolecule is desired (to impart two functions), the attachment must be made at a second site using currently available reagents. Since in some applications, it is desirable to have both an affinity ligand and an effector agent (e.g. a moiety that binds/bonds with a radionuclide), site-selective conjugation is precluded. Further, modification of biomolecules that are not made in a site-selective manner (e.g. reaction with surface amine groups in proteins) are limited due to the fact that two different sites are modified. Additionally,

modification of larger biomolecules (e.g. proteins) in two subsequent steps can result in a heterogeneous population of modified biomolecules in which molecules that contain the second conjugated species may have less of the desired biological properties (i.e. tumor targeting) than those that do not contain the second conjugate. This can result in a subgroup of biomolecules containing both conjugated species that do not have the properties desired. To circumvent these problems, the affinity ligand (e.g. biotin moiety) and an effector agent (e.g. radionuclide binding/bonding moiety with or without the radionuclide) can be coupled together through trifunctional cross-linking reagent to form a new type of reagent. With the use of this new class of reagents, an equal number of affinity ligands and radionuclide binding/bonding moieties will be conjugated to the biomolecule. With a combined affinity ligand and radiolabeling compound, site specific addition of both reagents can be made, and minimization of the number of conjugates to the biomolecule can be attained. Linking an affinity ligand such as biotin to a fluorescent moiety which is further attached to an oligosaccharide is described in Varki et al., WO 94/28008. The issue of attaching an affinity ligand to cytotoxic agent or an agent which can convert a prodrug to an active drug, and where either of these are further attached to a targeting molecule, is addressed in Nilsson et al., US patent application 08/090 047. However, none of these publications neither alone or in combination describe or indicate the present innovation. The issue of combining an affinity reagent and effector agent on one molecule to achieve minimal modification of biomolecules is not unique to biotin (as the affinity ligand) or radionuclide binding/bonding moieties (the effector

agent), and is not limited to only one affinity ligand and one effector ligand per molecule. Combinations of more than one affinity ligand and/or more than one affinity ligand per molecule may be advantageous for certain applications.

The radiolabeled and affinity ligand conjugated biomolecule products obtained from this invention are useful in many *in vitro* and *in vivo* applications. A preferred application, where the biomolecule is a tumor binding monoclonal antibody, toxin conjugate, or enzyme conjugate, the affinity ligand is biotin or a derivative thereof, and the radionuclide is a diagnostic or therapeutic radionuclide used in a patient cancer treatment protocol, is to use a biotin binding (e.g. avidin coated) column for extracorporeal immunoabsorptive removal of a radiolabeled antibody conjugate from a patient's blood. Extracorporeal removal of the radiolabeled antibody, toxin conjugate, or enzyme conjugate limits the toxic effects of the radioactivity, toxin, or enzyme to specifically targeted tissues, minimizing the exposure time and interaction with non-target tissues. Importantly, to be effective, medical agents (e.g. biomolecules) must exert their pharmacological action on a particular target tissue or group of target cells. Targeting of such agents is most often carried out by systemic administration (i.e. intravenous injection) which means that they will be transported through the blood and lymph system to most parts of the body. This transportation, or circulation, of the medical agent throughout the body can result in undesirable toxic side effects in tissues or organs other than those where the effect of the agents is beneficial to the patient.

Specific tissue or organ localization of a medical agent is a very important factor in its effective application. Lack of specific tissue localization is of particular importance in the treatment with medical agents where the desired effect is to kill certain types of cells such as in the treatment of cancer. In order to increase the specificity and thereby make the cancer therapy more effective, tumor marker specific targeting agents such as cancer cell binding monoclonal antibodies have been used as carriers for various cell toxic agents (immunoconjugates) such as, but not limited to, radionuclides, cytotoxins, and enzymes used in prodrug protocols (Meyer et al., Bioconjugate Chem. 6, 440-446, 1995; Houba et al., Bioconjugate Chem. 7, 606-611, 1996; Blakey et al., Cancer Res. 56, 3287-3292, 1996). Although, monoclonal antibodies are selectively bound with tumor cells over non-tumor cells, an initial high concentration of the toxic immunoconjugate is required to optimize binding of a particular agent with tumors in a patient. While required for optimal therapy of the cancer, the high concentration of cytotoxic material in blood and non-target tissues causes undesirable side-effects on sensitive and vital tissues like the bone marrow. Various methods have been proposed to rapidly clear these agents from blood circulation after that the tumor has received a maximum dose of the immunoconjugate. Some blood clearance methods involve the enhancement of the bodies own clearing mechanism through the formation of various types of immune complexes. Similarly, blood clearance can be obtained by using molecules that bind with the immunoconjugate, such as monoclonal antibodies (Klibanov et al., J. Nucl. Med. 29, 1951-1956, 1988; Marshall et al., Br. J. Cancer 69, 502-507, 1994; Sharkey et al. Bioconjugate Chem. 8, 595-

604, 1997), (strept)avidin (Sinitzyn et al., J. Nucl. Med. 30, 66-69, 1989; Marshall et al., Br. J. Cancer 71, 18-24, 1995), or biotin containing compounds which also contain sugar moieties recognized by the asialoglycoprotein receptor on liver cells (Ashwell and Morell, Adv. Enzymol. 41, 99-128, 1974). Other methods involve means of removing the circulating immunoconjugates through extracorporeal methods (see review article by Schriber G.J. & Kerr DE, Current Medicinal Chemistry, 1995, Vol. 2, pp 616-629).

The extracorporeal techniques used to clear a medical agent from blood circulation is particularly attractive. Extracorporeal devices for this application have been described (Henry CA, 1991, Vol.18, pp.565; Hofheinz D et al, Proc. Am. Assoc. Cancer Res. 1987 Vol. 28, pp. 391; Lear JL, et al. Radiology 1991, Vol.179, pp.509-512; Johnson TK, et al. Antibody Immunoconj. Radiopharm. 1991, Vol. 4, pp.509; Dienhart DG, et al. Antibody Immunoconj. Radiopharm. 1991, Vol. 7, pp.225 ; DeNardo GL, et al. J. Nucl. Med. 1993, Vol. 34, pp. 1020-1027 ; DeNardo GL, et al. J. Nucl. Med. 1992b, Vol. 33, pp. 863-864; DeNardo S.J., et. al. J. Nucl. Med. 1992a, Vol. 33, pp. 862-863. U.S. Patent Number 5,474,772; Australian patent 638061, EPO 90 914303.4 of Maddock, describe these methods.

To make the blood clearance more efficient and to enable processing of whole blood, rather than blood plasma, the medical agent (e.g. tumor specific monoclonal antibody carrying cell killing agents or radionuclides for tumor localization) have been biotinylated and cleared with the use of an affinity (e.g. biotin-binding) column. A number of publications provide data which show that this technique is both efficient and practical for the clearance of biotinylated and radionuclide labeled

tumor specific antibodies (Norrgren K, et al. Antibody Immunocnj Radiopharm 1991, Vol. 4, pp. 54 ; Norrgren K, et. al. J. Nucl. Med. 1993, Vol. 34, pp. 448-454 ; Garkavij M, et. al. Acta Oncologica 1996, Vol. 53, pp.309-312; Garkavij M, et. al. J. Nucl. Med. 1997, Vol. 38, pp. 895-901). U.S. Patent application No. 08/090,047, EPO 92 903 020.3 of Nilsson and 08/434,889 of Maddock describe these applications.

Summary of the Invention

10 The object of the present invention is to eliminate the above mentioned problems in the art. This object is achieved with a reagent as described by way of introduction and having the features defined by the characterising part of claim 1. Preferred embodiments are presented in the subclaims.

15 In general, the invention discloses a new type of compound which combines an affinity ligand and an effector agent in a single molecule that can be used to modify biomolecules. The modified biomolecules are themselves new entities in that fewer sites on them are modified than obtainable with previous reagents. More specifically, the invention describes the chemical components and examples of a new type of molecule (shown in schematic structure (I)) that can be used to conjugate an affinity ligand, such as biotin, and concurrently conjugate an effector ligand, such as a radionuclide binding/bonding moiety with/without a radiolabel, to a biomolecule of interest for a variety of diagnostic and therapeutic applications. This invention also discloses two approaches to the attaching both affinity ligands and radionuclides to a biomolecule (i.e. preformed and post-formed labeling approaches) in accordance to the routes shown in Scheme II. For therapeutic applications, a pre-

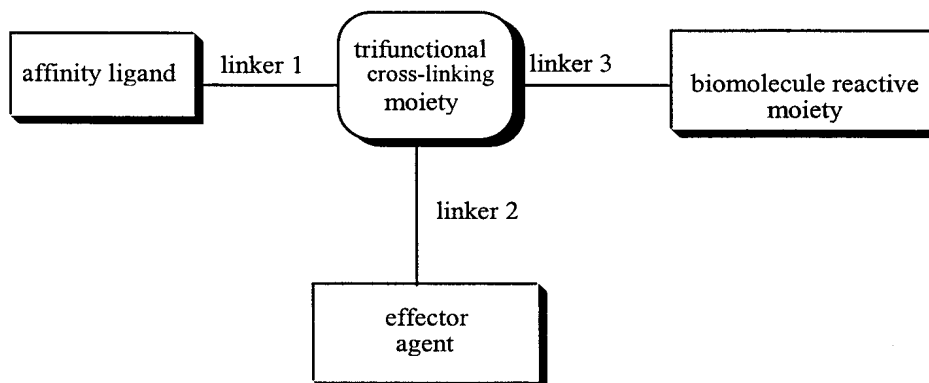
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ferred method of blood clearance of the new medical agent (conjugated biomolecule), using extracorporeal immunoabsorptive columns is disclosed.

Further, the new reagent according to the present invention can also be used for in vitro analysis of affinity labelled biomolecules, e.g. monoclonal antibodies or derivatives thereof, labelled with e.g. biotin or derivatives thereof. Thus, due to the presence of a photoactive agent, e.g. a chromophore or a fluorophore, as effector agent in the reagent molecule, it is possible to determine the amount of affinity label bound to the biomolecule as this amount is proportioned to the amount of photoactive agent.

Detailed Description

General structure of compounds disclosed. The chemical nature of a compound for concurrent conjugations of an affinity ligand and an effector agent is shown graphically in the schematic structure (I). A brief description of the various parts of the generalized formulation is provided in the text following the schematic structure (I):



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The term "affinity ligand" used throughout the description and the claims means any moiety that binds with another molecule with an affinity constant of 10^6 M⁻¹ or higher. A preferred affinity ligand is a biotin moiety which can be biotin, or any derivative or conjugate of biotin that binds with avidin, streptavidin, or any other biotin binding species.

The term "effector agent" used throughout the description and the claims means a radionuclide binding moiety with or without the radionuclide, a synthetic or naturally occurring toxin, an enzyme capable of converting pro-drugs to active drugs, immunosuppressive or immunostimulating agents, or any other molecule known or found to have a desired effect, directly or indirectly, on cells or tissues.

The term "biomolecule reactive moiety" used throughout the description and the claims means any moiety that will react with a functional group naturally occurring or synthetically introduced on a biomolecule.

The term "trifunctional cross-linking moiety" used throughout the description and the claims means any chemical moiety that can combine the affinity ligand (e.g. biotin moiety), effector agent (e.g. radionuclide binding/bonding moiety) and a biomolecule reactive moiety.

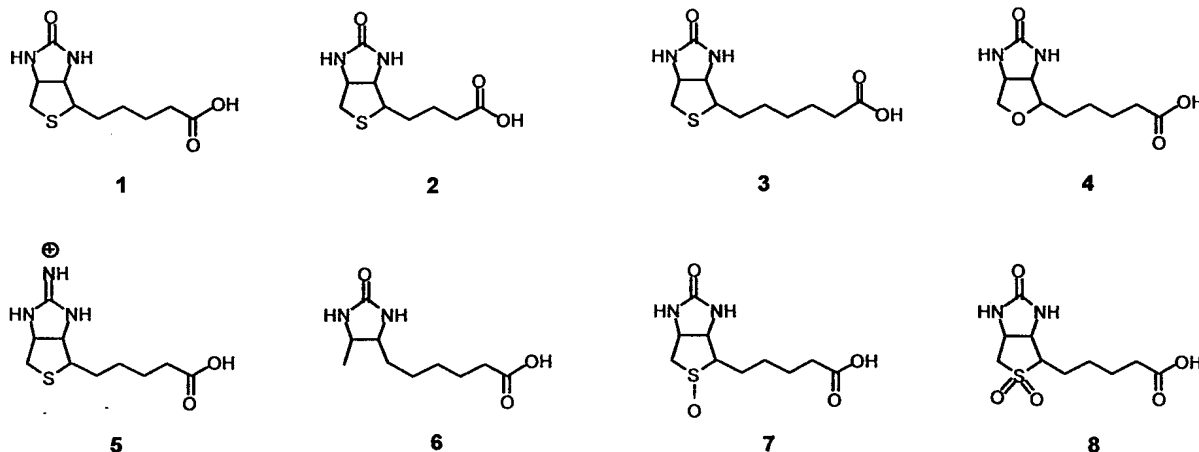
The term "linker 1" used throughout the description and the claims means a chemical moiety that is an attaching moiety and spacer between the trifunctional cross-linking moiety and the biotin moiety such that binding with avidin or streptavidin, or any other biotin binding species, is not diminished by steric hindrance. Linker 1 may also impart increased water solubility and biotinidase stabilization.

The term "linker 2" used throughout the description and the claims means a chemical moiety that is used to attach the radionuclide binding moiety to the trifunctional cross-linking moiety. Linker 2 may also impart increased water solubility.

The term "linker 3" used throughout the description and the claims means a chemical moiety used to attach the biomolecule reactive moiety to the trifunctional cross-linking moiety. Linker 3 may not be required, but may be advantageous in some cases. Linker 3 may be used as a spacer and/or it may be used to increase the water solubility of the compound.

Affinity ligand . The preferred affinity ligand is biotin or a derivative thereof. In most examples the biotin moiety will be natural biotin 1, which is coupled to linker 1 through an amide bond. In some examples it may be advantageous to have a biotin derivative that does not bind as tightly as natural biotin, or a biotin derivative that binds to chemically modified, or genetically mutated, avidin or streptavidin in preference to natural biotin. Examples of such biotins are norbiotin 2, homobiotin 3, oxybiotin 4, iminobiotin 5, desthiobiotin 6, diaminobiotin 7, biotin sulfoxide 8, and biotin sulfone 9. Other modifications of biotin, including further modification of 2 - 9, are also included.

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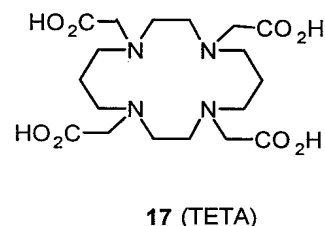
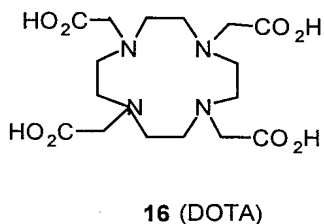
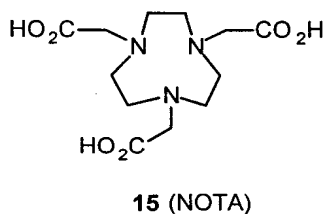
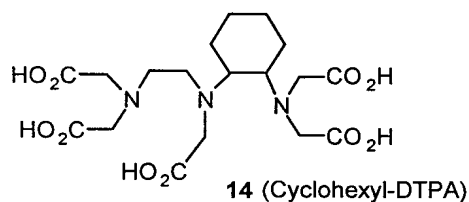
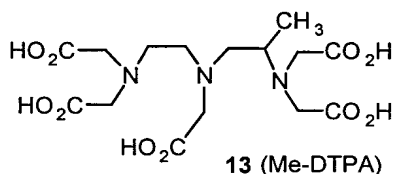
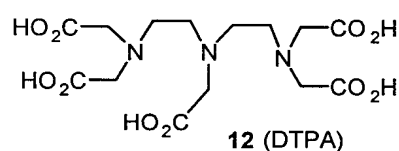
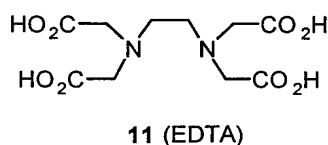
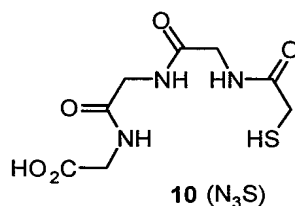
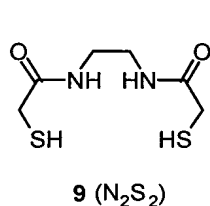


Effector agent. The preferred effector agent is a radionuclide binding/bonding moiety, with or without the radionuclide being present. There are a large number of radionuclides that are potentially useful for diagnostic and therapeutic purposes (see articles in Spencer et al. eds., *Radionuclides in Therapy*, CRC Press, 1987; Ruth et al., *Nucl. Med. Biol.* 16, 323-336, 1989), and thus moieties which bind or bond with them may be incorporated as the radionuclide binding/bonding moiety. Examples of gamma imaging radionuclides include, Tc-99m, In-111, and I-123. Examples of positron imaging radionuclides include Ga-68, F-18, Br-75, Br-76, and I-124. Examples of therapeutic radionuclides include Y-90, I-131, Re-186, Re-188, Cu-67, Sm-153, Lu-177, Bi-212, Bi-213 and At-211. It is a requirement that the radionuclides be bound by chelation (for metals) or covalent bonds in such a manner that they do not become separated from the biotinylation/- radiolabeling compound under the conditions that the biomolecule conjugates are used (e.g. in patients). Thus, the most stable chelates or covalent bonding arrangements are preferred. Examples of such binding/bonding moieties are: aryl halides and vinyl halides for radionuclides of halogens; N_2S_2 9 and N_3S 10 chelates

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for Tc and Re radionuclides; amino-carboxy derivatives such as EDTA 11, DTPA 12, derivatives Me-DTPA 13 and cyclohexyl-DTPA 14, and cyclic amines such as NOTA 15, DOTA 16, TETA 17, CITC-DTPA (not shown, US patent 4,622,420), and triethylenetetraaminehexaacetic acid derivatives (not shown, see Yuangfang and Chuanchu, Pure & Appl. Chem. 63, 427-463, 1991) for In, Y, Pb, Bi, Cu, Sm, Lu radionuclides. Attachment of the radionuclide binding/bonding moiety to linker 2 can be achieved at a number of locations in the moieties.

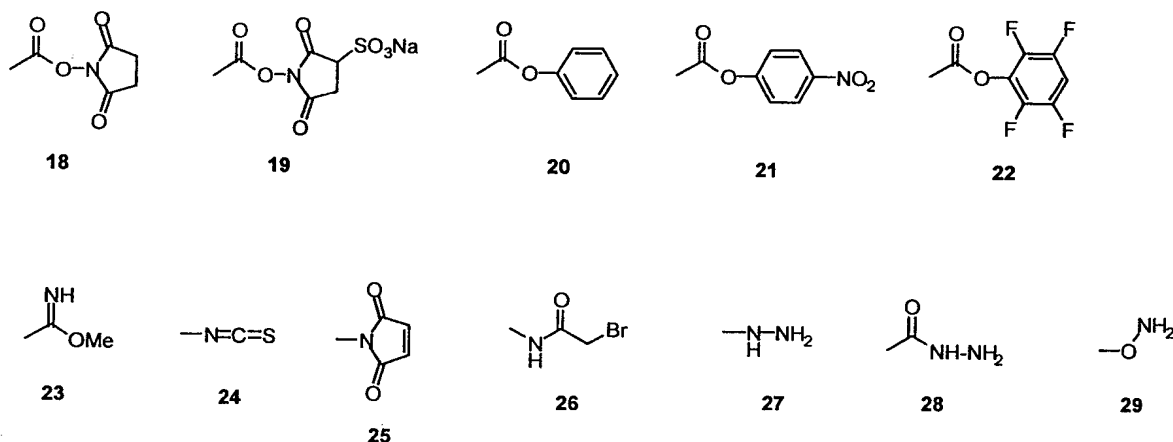
The effector agent can also be a photoactive compound or a compound which can be converted to a photoactive compound, such as a chromophore, fluorophore or any other conventionally used photoactive compound.



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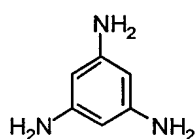
Biomolecule reactive moiety. There are a number of moieties that are reactive with functional groups that may be present on a biomolecule, e.g. a protein. For example, aryl or alkyl activated carboxylic acids can be reacted with nucleophilic groups such as primary or secondary amines. Such activated esters include: N-hydroxysuccinimide esters 18, sulfo-N-hydroxysuccinimide esters 19, phenolic esters (e.g. phenol 20, p-nitrophenol 21, tetrafluorophenol 22). Other amine reactive groups include aryl and alkyl imidates 23 and alkyl or aryl isocyanates or isothiocyanates, 24. Sulfhydryl groups on the biomolecule can be reacted with maleimides 25 or alpha-haloamide 26 functional groups. Biomolecules containing naturally occurring or synthetically produced (e.g. by conjugation or from oxidized sugar moieties) aldehydes and ketones can be reacted with aryl or alkyl hydrazines 27, aryl or alkyl acylhydrazines 28, alkyl or aryl hydroxylamines 29.

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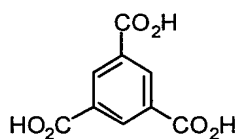


Trifunctional cross-linking moiety. The trifunctional cross-linking moiety has two functional groups that can be used to couple with linker 1 and linker 2. It

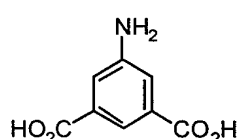
has another functional group that can be either converted directly into the biomolecule reactive moiety or coupled with linker 3. Examples of preferred trifunctional cross-linking moieties are triaminobenzene 30, tricarboxybenzene 31, dicarboxyaniline 32, and diaminobenzoic acid 33. If the functional groups present on the cross-linking



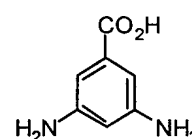
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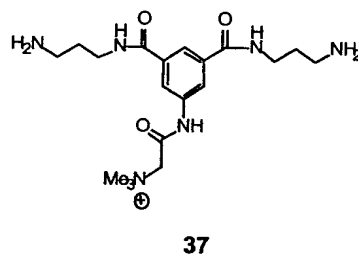
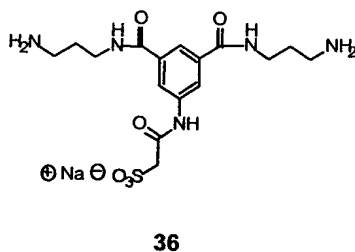
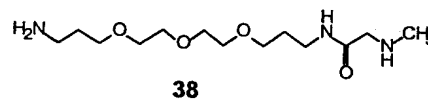
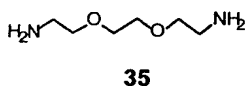
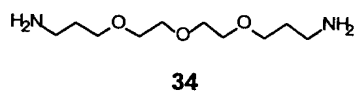
moiety are not by themselves reactive with a functional group on the biomolecule, then they are converted into more reactive moieties, such as activated esters (for carboxylic acids), imidates (cyano functional groups), maleimides (amino), isocyanates, isothiocyanates, etc. The functional groups present on the cross-linking moiety may vary, and protection/deprotection/activation steps may be required to synthesize the desired compound. A trifunctional cross-linking moiety is preferred, but in those examples where more than one effector agent, affinity ligand, or protein reactive moiety is advantageous, tetrafunctional, or higher, cross-linking moieties may be applied.

Linker moieties. The linker moieties function as spacers and also may aid in water solubilization for compounds that do not contain ionized or ionizable functionalities. Linker 1 must provide ample space between the biotin moiety and the trifunctional cross-linking moiety such that there is a minimum of 9Å for biotin binding with avidin or streptavidin. Extended linkers (e.g. 6 - 20 atoms in length) are preferred to assure

that there is no steric hindrance to binding avidin or streptavidin from the biomolecule that the conjugate is attached to. The extended linkers may contain hydrogen bonding atoms such as ethers or thioethers, or ionizable groups such as carboxylates, sulfonates, or ammonium groups, to aid in water solubilization of the biotin moiety. Many of the biotin moieties are highly insoluble in water. When the compounds of this invention are used in serum or in animals or people, there is an additional requirement for a linker attached to biotin that is not required for linkers attached to other moieties. This requirement is to provide a means of blocking the enzyme biotinidase (Wolf et al., *Methods Enzymol.* 184, 103-111, 1990; Pipsa, *Ann. Med. Exp. Biol. Fenn* 43, Suppl. 5, 4-39, 1965) from cleaving the amide bond (biotinamide) to release biotin. This requirement is met by altering the distance between the bicyclic rings of the biotin moiety (as in norbiotin or homobiotin) or using a biotin derivative that has a dramatically decreasing binding with avidin or streptavidin (e.g. desthiobiotin). If natural biotin is used, blockade of biotinidase activity is provided by introducing an alpha carboxylate (Rosebrough, *J. Pharmacol. Exp. Ther.* 265, 408-415, 1993) or an N-methyl group (Wilbur et al., *Bioconjugate Chem.* 8, 572-584, 1997) in Linker 1.

Linker 2 must provide a means of coupling an effector agent, such as a radionuclide binding/bonding moiety, with the trifunctional cross-linking moiety. The nature of linker 2 can be highly dependent on the chemistry associated with effector agent employed, particularly in the case where the effector agent is a radionuclide binding/bonding moiety. Although linker 2 may be as short as 1 atom, it is preferred to have more space than 1 atom

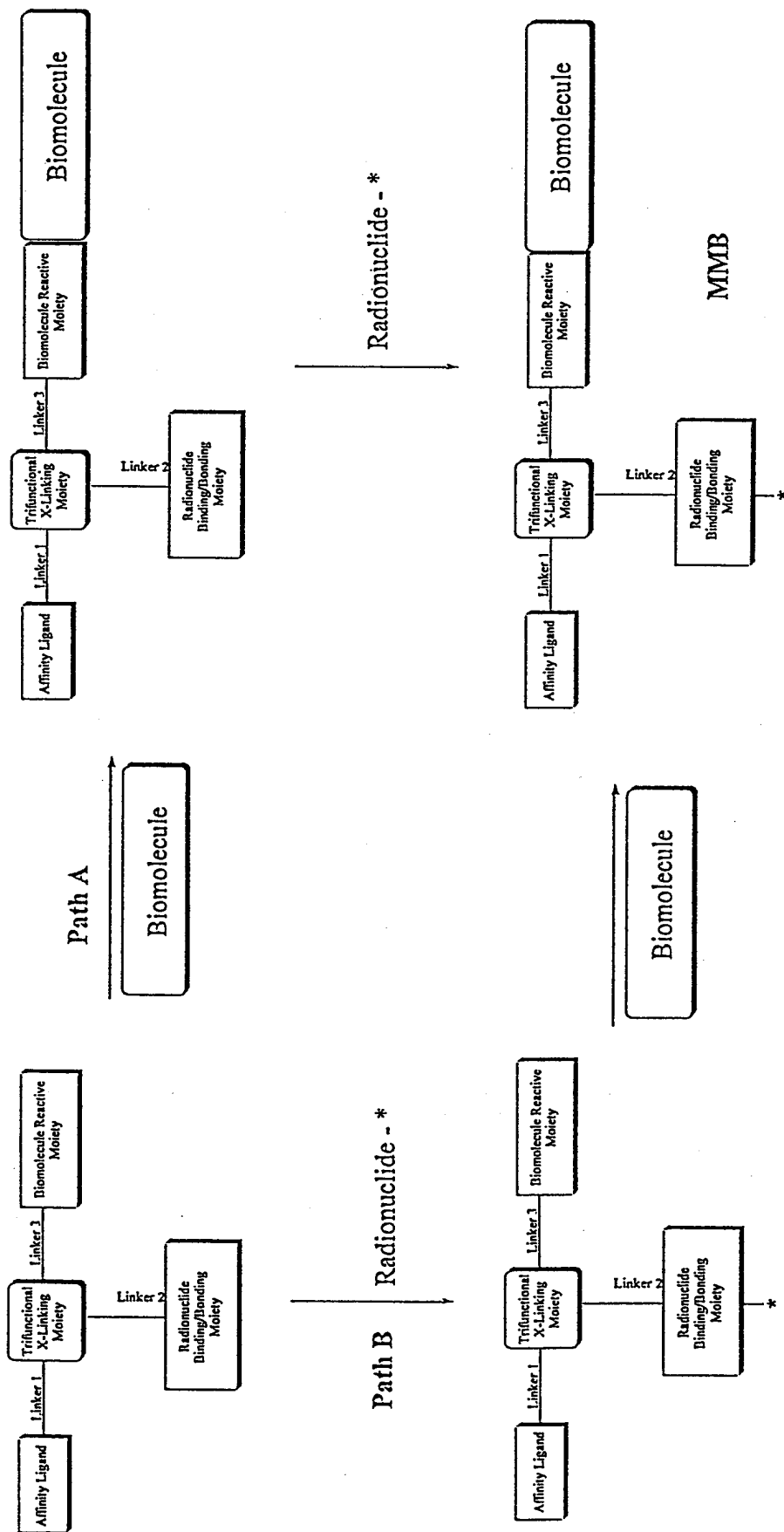
provided to decrease the steric environment around the affinity ligand (e.g. biotin moiety). Linker 2 can also have the water solubilizing atoms or groups of atoms to increase water solubility. Linker 3, if required, provides additional space between the biomolecule and the biotin moiety, and can be used to provide additional water solubilization where required. Examples of preferred non-ionized linkers include the trioxadamine 34 and dioxadamine 35. Examples of preferred ionized linkers include aryl diaminosulfonate 36 and aryl diamino-trimethylammonium 37. Examples of linkers that also contain a biotinidase blocking moiety are made by combining one of the linkers 34 - 37 with another molecule, for example combining linker 34 with N-methylglycine to yield linker 38, where the N-methyl end must be attached to the biotin moiety to impart stability towards biotinidase cleavage.



This invention discloses new chemical species that are composed of any combination of affinity ligands (e.g. biotin moieties), effector agents (e.g. radionuclide binding moieties), protein reactive moieties, trifunctional cross-linking moiety, and linking moieties. In

specific examples, the reagents of this invention (generically shown in schematic structure (I)) provide a means of biotinylation and radiolabeling of biomolecules. This results in a minimally modified biomolecule (MMB). Irrespective of the individual components of the new chemical species, the process of conjugation and radiolabeling can occur by two distinctly different methods to give the same final product (the MMB), as depicted in Scheme (II) below. Path A is termed postformed conjugate (radio)labeling and Path B is termed preformed conjugate (radio)labeling. Path A, where a compound of this invention is conjugated with the biomolecule first, and subsequently radiolabeled with the radionuclide chosen, is the preferred method of conjugation and radiolabeling. However, some radionuclide binding/bonding conditions are not compatible with certain biomolecules, therefore, Path B may be used as an alternative approach.

Scheme 2 Radiolabeling and Biomolecule Conjugation Paths



EXAMPLES

The following examples 1-7 are provided to show some of the different combinations of reagents that are disclosed herein, and to show methods for preparing them. The examples are provided by way of illustration, not by way of limitation. Many further examples can be made by differing combinations of chemical moieties as depicted in the general formulation. The examples 1-6 are followed by reaction schemes relating to each example for the production of the reagents 39-44 according to the present invention.

Example 1

Compound 39 is a reagent according to the present invention and contains biotin as the biotin moiety; a biotinidase stabilized linker as linker 1; aminoisophthalic acid as the trifunctional cross-linking moiety; a CHX-DTPA group as a chelator for In-111 and Y-90; an aminobenzyl group for linker 2; no linker 3; and an isothiocyanate biomolecule reactive moiety. A method for synthesizing 39 from previously known reagents is provided.

Example 2

Compound 40 is a reagent according to the present invention and contains biotin as the biotin moiety; a biotinidase stabilized (N-methyl) linker as linker 1; aminoisophthalic acid as the trifunctional cross-linking moiety; a tri-n-butylstannylbenzoate group as a moiety that is rapidly reacted to bond with the radiohalogens Br-75/76/77, I-123/124/125/131, or At-211; a trioxadiazine for linker 2; no linker 3; and a tetrafluorophenyl

ester biomolecule reactive moiety. A method for synthesizing 40 from previously known reagents is provided.

Example 3

5 Compound 41 is a reagent according to the present invention and contains homobiotin as the biotin moiety; a trioxadiazine linker as linker 1; aminoisophthalic acid as the trifunctional cross-linking moiety; an acid labile protected N_2S_2 group as a chelator for Tc-99m or Re-
10 186/188; an propionate moiety for linker 2; no linker 3; and a tetrafluorophenyl ester biomolecule reactive moiety. A method for synthesizing 41 from previously known reagents is provided.

15 Example 4

 Compound 42 is a reagent according to the present invention and contains homobiotin as the biotin moiety; a trioxadiazine linker as linker 1; aminoisophthalic acid as the trifunctional cross-linking moiety; a BAT group as
20 a chelator for Tc-99m or Re-186/188; a pentyloxybenzoate group for linker 2; no linker 3 and a tetrafluorophenyl ester biomolecule reactive moiety. This example is shown in that the BAT chelate allows the reagent to be coupled with a biomolecule (e.g. protein) prior to attaching the
25 radionuclide. Modification Path A. A method for synthesizing 42 from previously known reagents is provided.

Example 5

 Compound 43 is a reagent according to the present
30 invention and contains biotin as the biotin moiety; a biotinidase stabilized linker as linker 1; aminoisophthalic acid as the trifunctional cross-linking moiety; a TETA group as a chelator for Cu-67; an ami-

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benzyl group for linker 2; no linker 3; and an isothiocyanate biomolecule reactive moiety. A method for synthesizing 43 from previously known reagents is provided.

5

Example 6

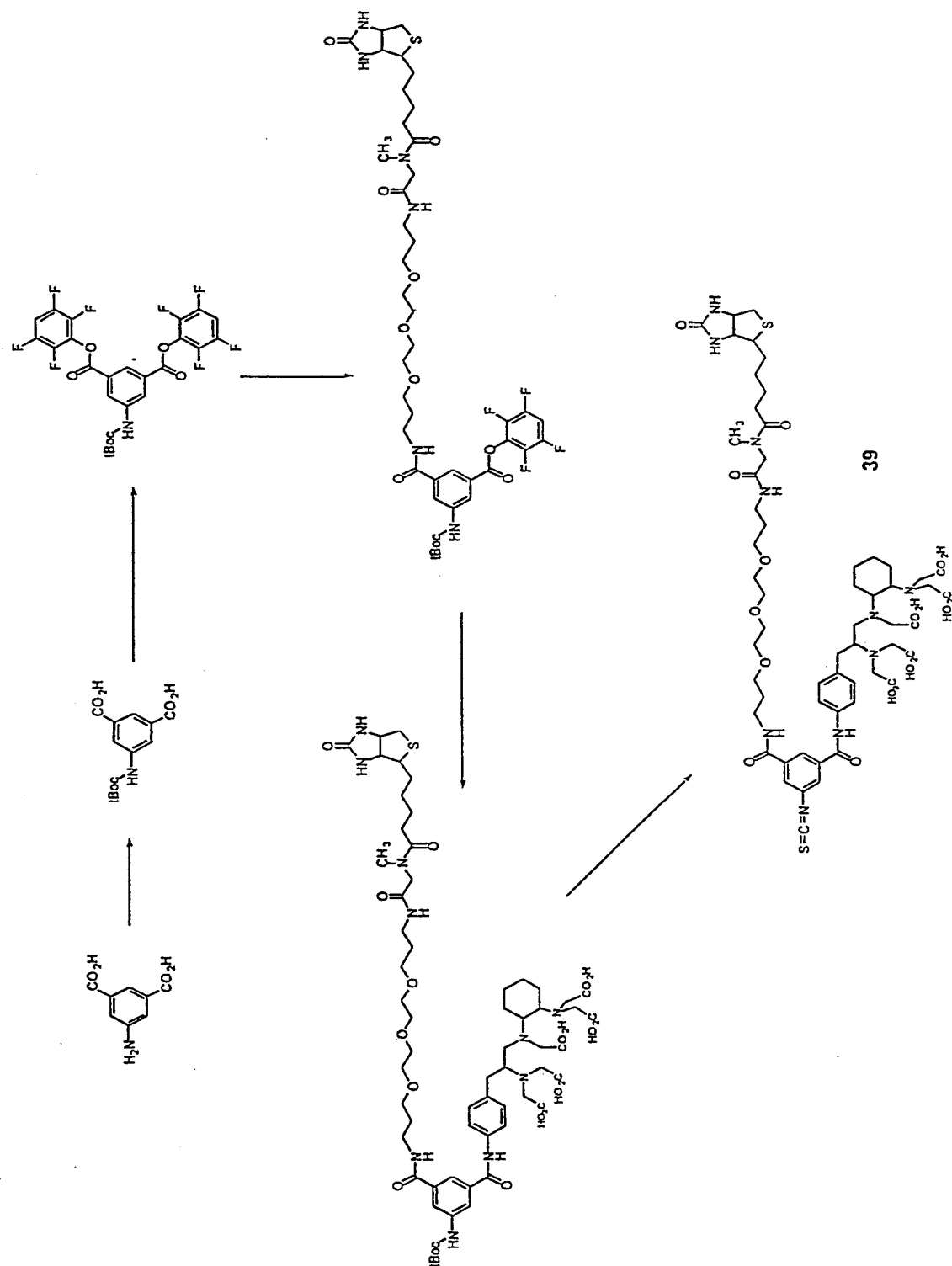
Compound 44 is a reagent according to the present invention and contains biotin as the biotin moiety; a biotinidase stabilized linker as linker 1; tricarboxybenzene as the trifunctional cross-linking moiety; a tri-n-butylstannylbenzoate moiety for reaction with radiohalogens; a trioxadiazine moiety for linker 2; a trioxadiazine moiety for linker 3; and a maleimide group as the biomolecule reactive moiety. A method for synthesizing 44 from previously known reagents is provided.

Example 7

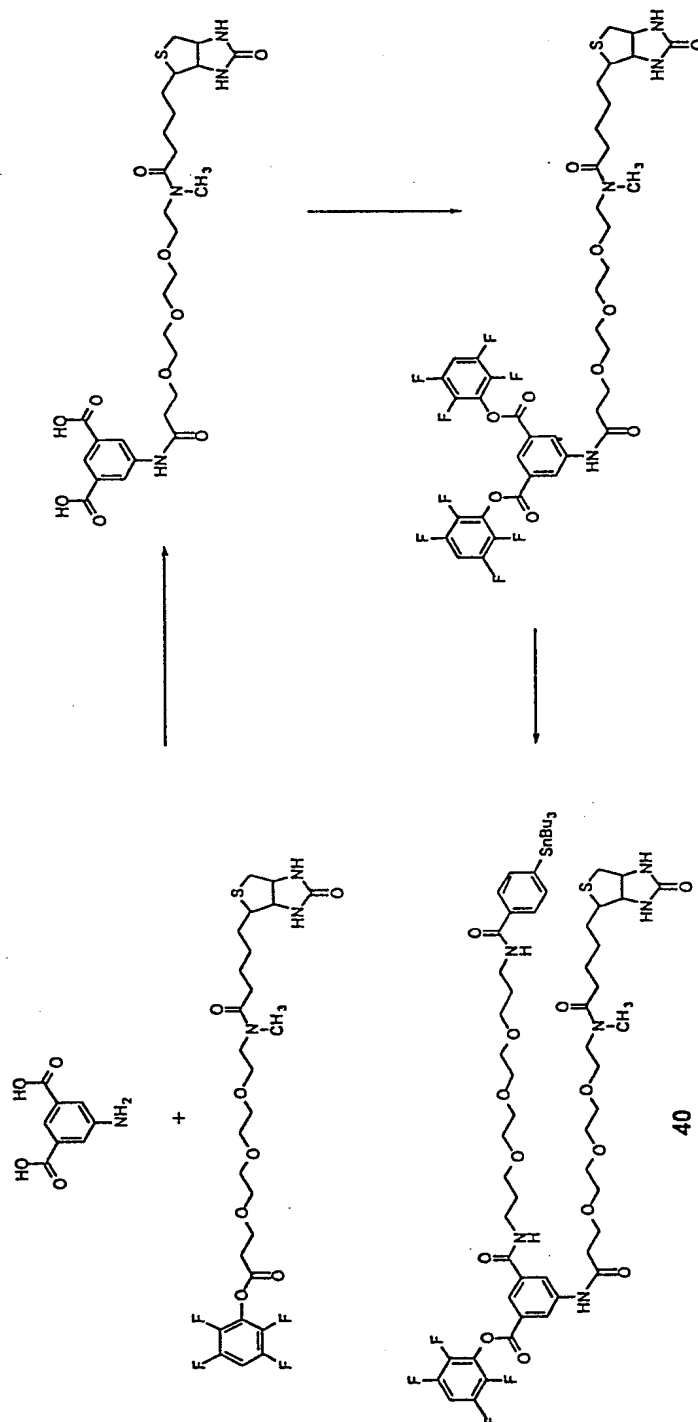
Compound 45 is a reagent according to the present invention and contains biotin as the biotin moiety; a biotinidase stabilized linker (the glycyl moiety is replaced by an aspartyl moiety as linker 1; amino-isophthalic acid as the trifunctional cross-linking moiety; a CHX-A"-DTPA group as a chelator for In-111, Y-90 and Bi-213; an aminobenzyl group for linker 2; no linker 3; and an isothiocyanate biomolecule reactive moiety. The synthesis sequence of reactions to prepare this compound are shown in scheme 7.

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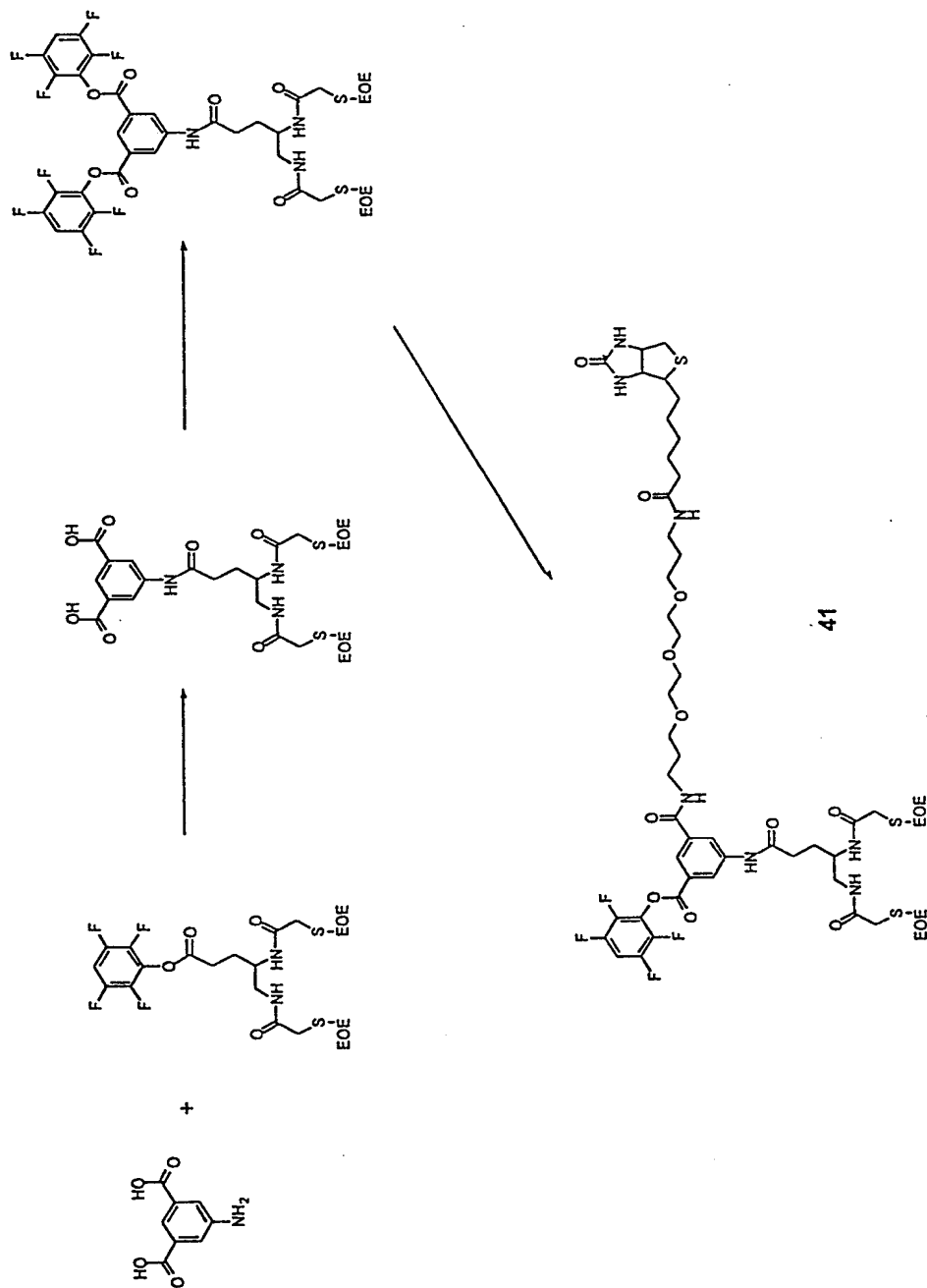
Example 1
Reagent with Biotin, Biotinidase Stabilizing Linker, CHX-DTPA Chelate, and Isothiocyanate



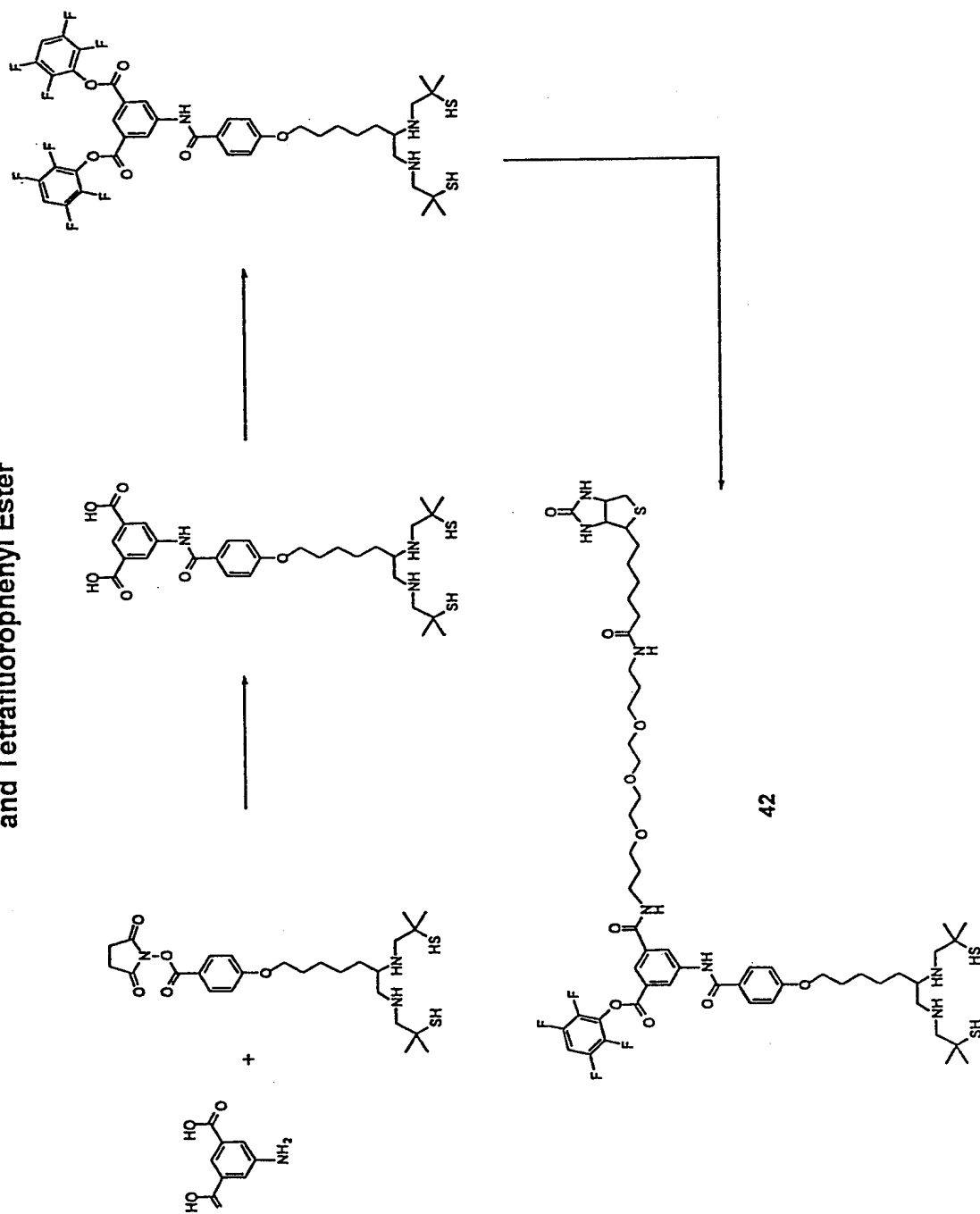
Example 2
 Reagent with Biotin, Biotinidase Stabilized Linker, Arylstannane
 Radiohalogenation Moiety, and Tetrafluorophenyl Ester



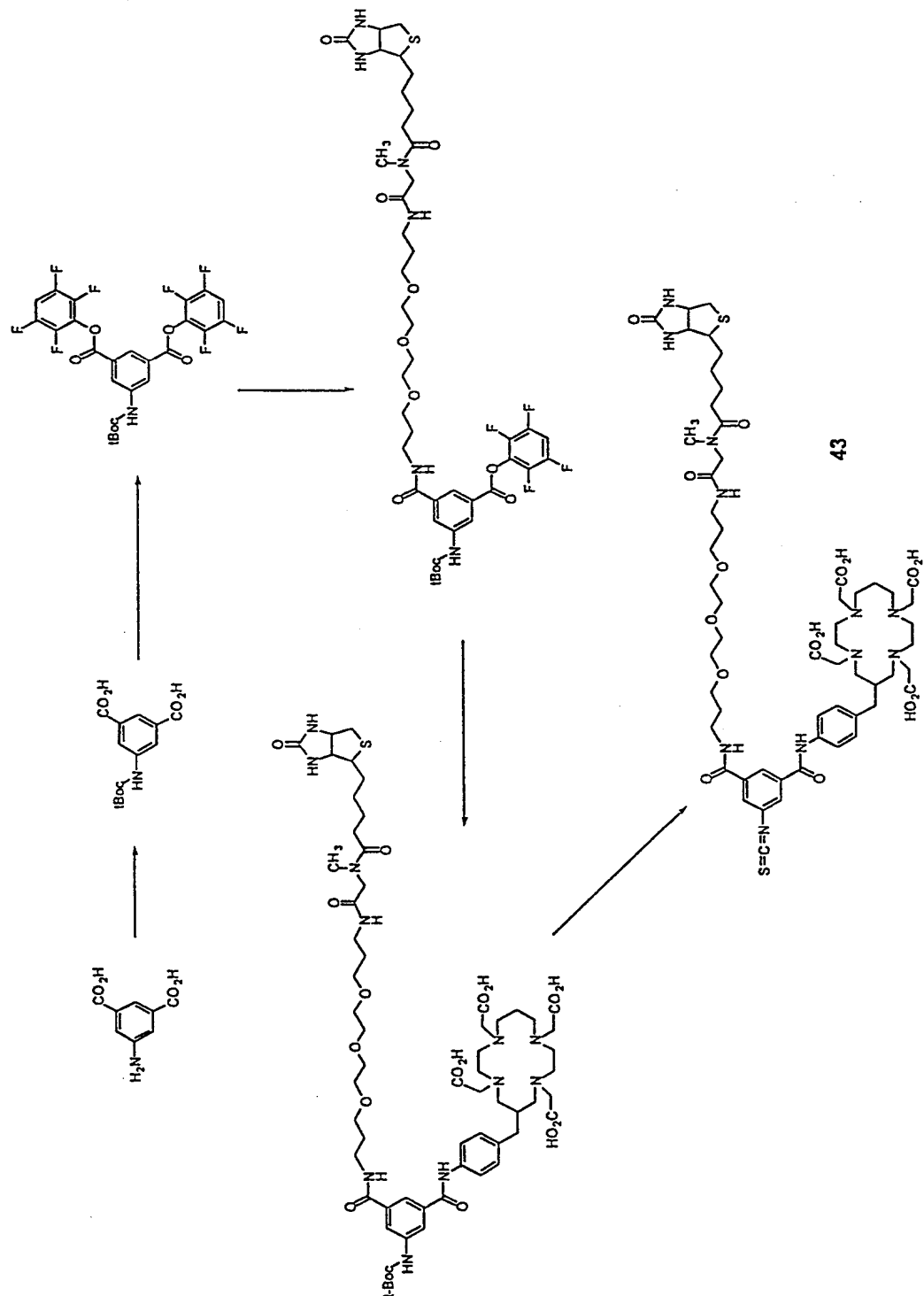
Example 3
Reagent with Homobiotin, Diamidodithio (N_2S_2) Chelate, and
Tetrafluorophenyl Ester



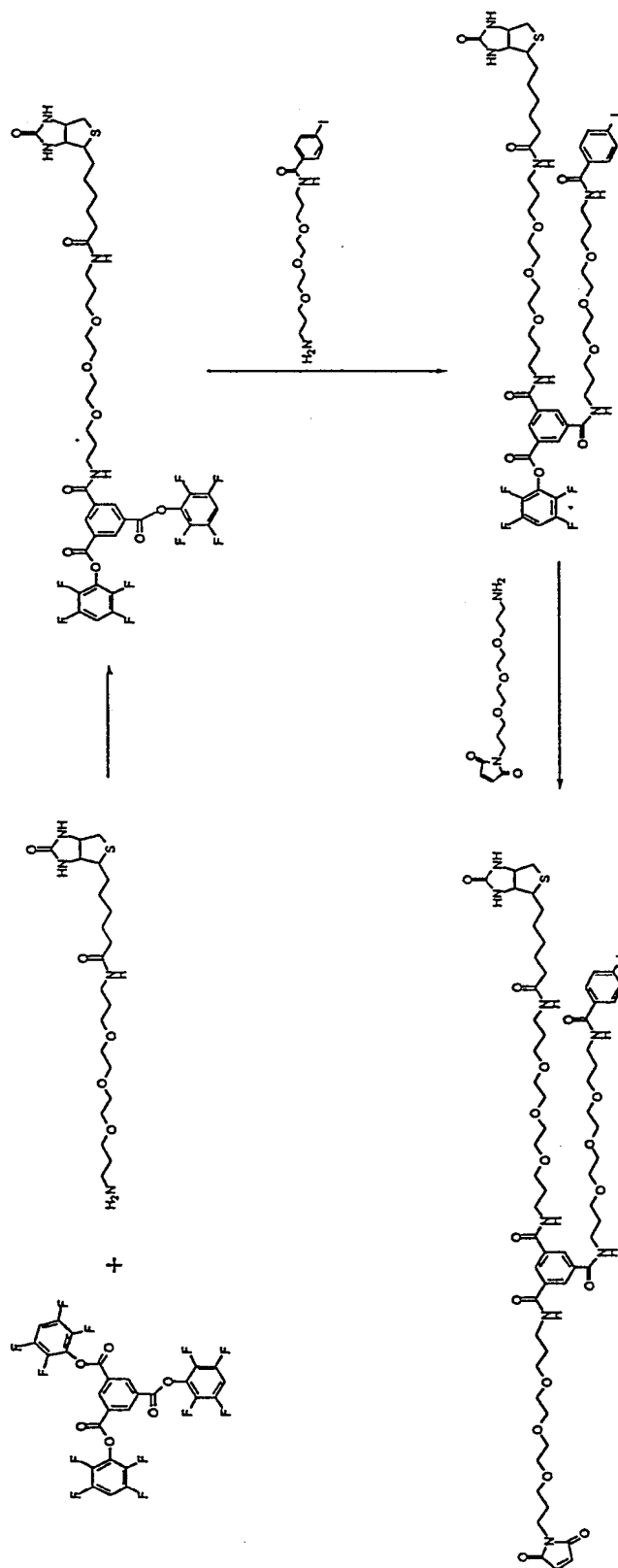
Example 4
Reagent with Homobiotin, DiaminoDithio (N_2S_2) Chelate,
and Tetrafluorophenyl Ester



Example 5
Reagent with Biotin, Biotinidase Stabilizing Linker,
TETA Chelate, and Isothiocyanate

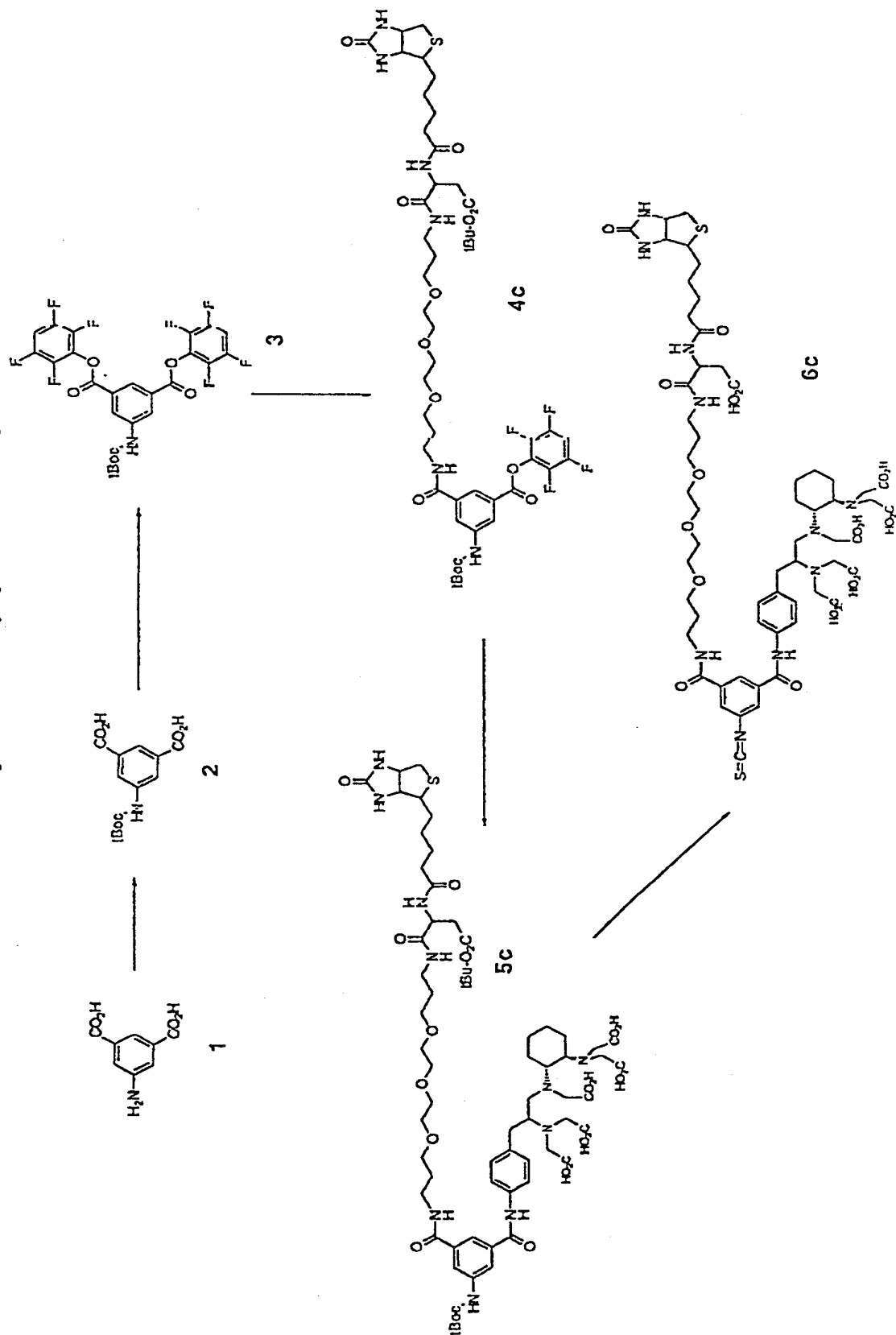


Example 6
Reagent with Homobiotin, Arylstannyl Radiohalogenation Moiety, and Maleimide



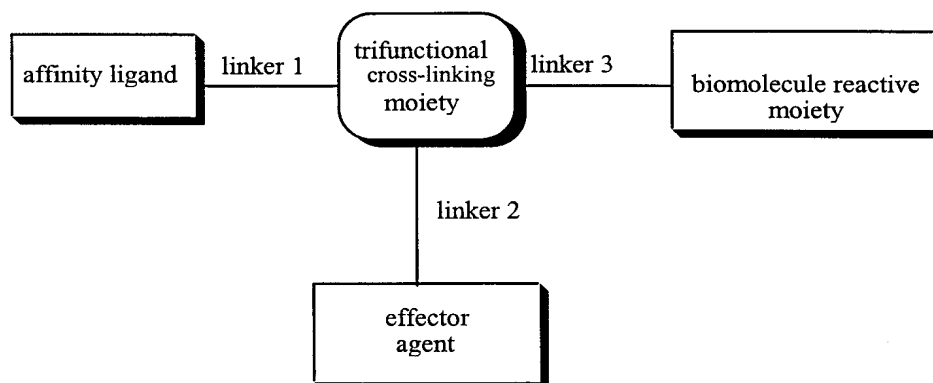
Exempel 7

Reagent with Biotin, Biotinidase Stabilizing Linker, CHX-A"-DTPA Chelate, and Isothiocyanate Conjugation Moiety



CLAIMS

1. Reagent for conjugation to a biomolecule, wherein
 5 the reagent is a single molecule with at least three functional parts and has the following schematic structure (I):



10

a) wherein a trifunctional cross-linking moiety is coupled to

b) an affinity ligand via a linker 1, said affinity ligand being capable of binding with another
 15 molecule having affinity for said ligand, to

c) an effector agent, optionally via a linker 2, said effector agent exerting its effect on cells, tissues and/or humorous molecules in vivo or ex vivo, and to

20 d) a biomolecule reactive moiety, optionally via a linker 3, said moiety being capable of forming a bond between the reagent and the biomolecule.

2. Reagent according to claim 1, wherein the trifunctional cross-linking moiety is chosen from the group
 25 consisting of triaminobenzene, tricarboxybenzene, dicarboxyaniline and diaminobenzoic acid.

3. Reagent according to claims 1 and 2, wherein the affinity ligand is a moiety that binds with another molecule with an affinity constant of 10^6 M^{-1} or higher.

4. Reagent according to claims 1-3, wherein the
5 affinity ligand is a moiety which binds specifically to avidin, streptavidin or any other derivatives, mutants or fragments of avidin or streptavidin having essentially the same binding function to the affinity ligand.

5. Reagent according to claims 1-4, wherein the
10 affinity ligand is biotin, or a biotin derivative having essentially the same binding function to avidin or streptavidin as biotin.

6. Reagent according to claims 1-5, wherein the biotin derivative is chosen from the group consisting of
15 norbiotin, homobiotin, oxybiotin, iminobiotin, desthio-biotin, diaminobiotin, biotin sulfoxide, and biotin sulfone, or other molecules thereof that having essentially the same binding function.

7. Reagent according to claim 5, wherein the sta-
20 bility towards enzymatic cleavage, preferably by biotinidase, of the biotinamide bond to release biotin has been improved by using biotin derivatives, preferably norbiotin or homobiotin.

8. Reagent according to claims 1-6, wherein linker 1
25 serves as an attaching moiety and a spacer between the trifunctional cross-linking moiety and the biotin moiety such that binding with avidin or streptavidin, or any other biotin binding species, is not diminished by steric hindrance.

30 9. Reagent according to claims 1-8, wherein linker 1 contains hydrogen bonding atoms such as ethers or thioethers, or ionizable groups such as carboxylates, sulfon-

ates, or ammonium groups to aid in water solubilization of the biotin moiety.

10. Reagent according to claims 1-9, wherein stability towards enzymatic cleavage, preferably by biotinidase, of the biotinamide bond to release biotin have been improved by introducing an alpha carboxylate or an N-methyl group in linker 1.

11. Reagent according to claim 1, wherein the effector agent is chosen from the group consisting of synthetic or natural occurring toxins, enzymes, preferably enzymes capable of converting a pro-drug to an active drug, hormones, immunosuppressive agents, immunostimulating agents, radionuclide binding/bonding moieties, radiosensitizers, enhancers for X-ray or MRI or ultrasound, non-radioactive elements which can be converted to radioactive elements by means of external irradiation after that the biomolecule carrying said element has been accumulated to specific cells or tissues, or compounds used in photoimaging or photodynamic therapy.

12. Reagent according to claims 1-11, wherein the effector agent is a radionuclide binding/bonding moiety to which radionuclides can be bound by chelation or covalent bonding.

13. Reagent according to claim 7, wherein the effector agent is a radionuclide binding/bonding moiety to which radionuclides are bound by chelation or through covalent bonding.

14. Reagent according to claims 1-13, wherein the effector agent comprises aryl halides and vinyl halides for radionuclides of halogens, amino-carboxy derivatives, preferably EDTA and DTPA derivatives, including Me-DTPA, CITC-DTPA, and cyclohexyl-DTPA, and cyclic amines, pre-

ferably NOTA, DOTA, and TETA for In, Y, Pb, Bi, Cu, Sm, and Lu radionuclides.

15 15. Reagent according to claims 1-14, wherein the effector agent is provided with positron imaging radionuclides, preferably F-18, Br-75, Br-76, and I-124; therapeutic radionuclides, preferably Y-90, I-131, In-114m, Re-186, Re-188, Cu-67, Sm-157, Lu-177, Bi-212, Bi-213, At-211, Ra-223; and gamma imaging radionuclides, preferably Tc-99m, In-111 and I-123.

10 16. Reagent according to claims 1-11, wherein the effector agent is a photoactive compound or a compound which can be converted to a photoactive compound, preferably a chromophore or fluorophore or alike compound.

15 17. Reagent according to claims 1-16, wherein linker 2 is excluded.

18. Reagent according to claims 1-16, wherein linker 2 provides a spacer length of 1-25 atoms, preferably a length of 6-18 atoms, or groups of atoms.

20 19. Reagent according to claims 1-16, and 18, wherein linker 2 contains hydrogen bonding atoms, preferably ethers or thioethers, or ionizable groups, preferably carboxylates, sulfonates, or ammonium groups, to aid in water solubilization.

25 20. Reagent according to claims 1-19, wherein the biomolecule reactive moiety is chosen from the group consisting of active esters, preferably N-hydroxy-succinimide esters, sulfo-N-hydroxysuccinimide esters, phenolic esters, aryl and alkyl imitates, alkyl or aryl isocyanates or isothiocyanates reacting with amino groups
30 on the biomolecule, or maleimides or alpha-haloamides reacting with sulfhydryl groups on the biomolecule, or aryl or alkylhydrazines or alkyl or aryl hydroxylamines

reacting with aldehyde or ketone groups naturally occurring or synthetically produced on the biomolecule.

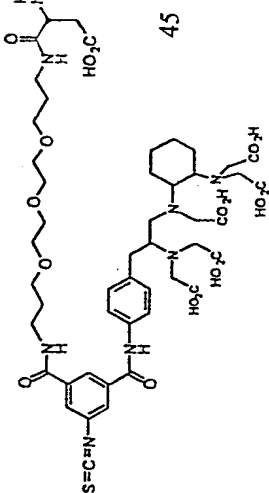
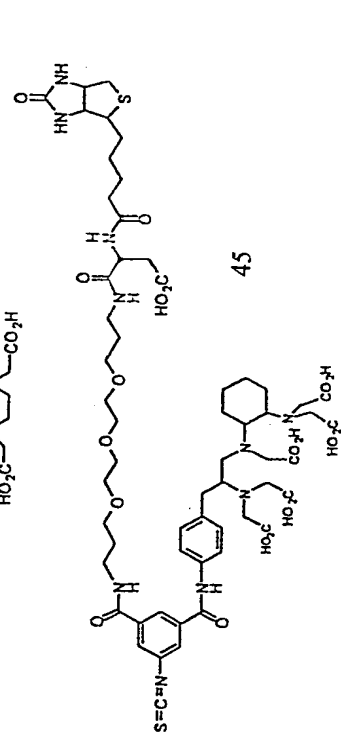
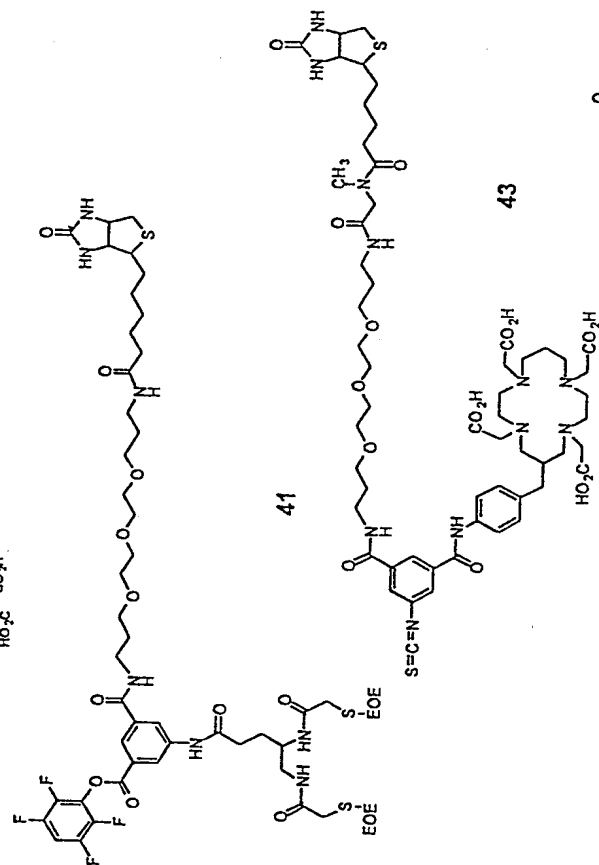
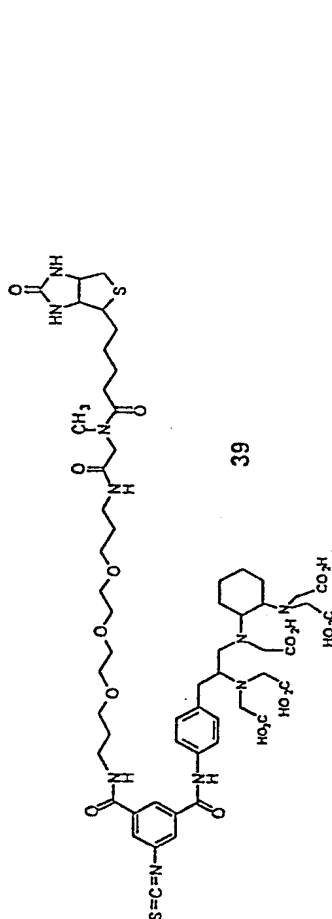
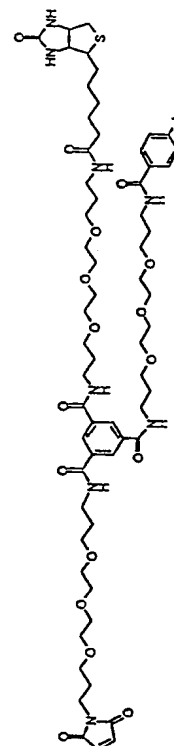
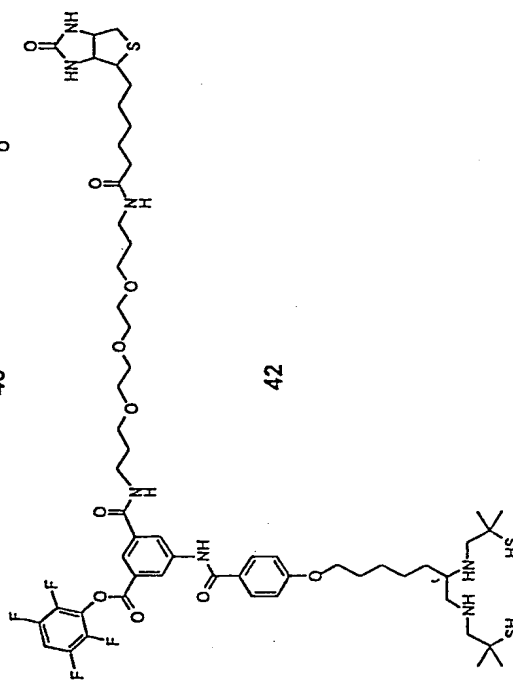
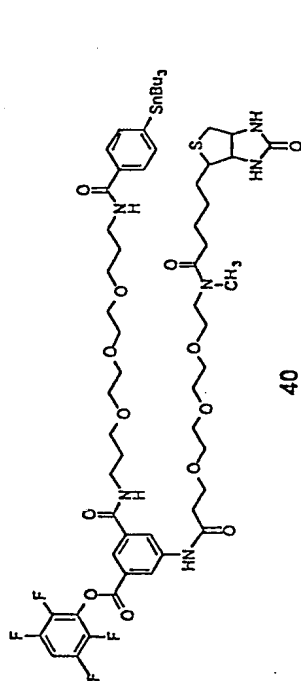
21. Reagent according to claims 1-20, wherein linker 3 is excluded.

5 22. Reagent according to claims 1-20, wherein linker 3 provides a spacer of a length of 1-25 atoms, preferably a length of 6-18 atoms, or groups of atoms.

23. Reagent according to claims 1-20 and 22, wherein linker 3 contains hydrogen bonding atoms such as ethers
10 or thioethers, or ionizable groups, preferably as carboxylates, sulfonates, or ammonium groups to aid in water solubilization.

24. Reagent according to any of the previous claims, wherein it is chosen from the group consisting of the
15 following compounds:

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25. Reagent according to claim 1, wherein more than one affinity ligand and/or more than one effector agent are bound to a trifunctional or tetrafunctional cross-linking group.

5 26. Reagent according to any of the previous claims for diagnosis and treatment of human and animal conditions or diseases, preferably in targeting of cancer, myocardial infarcts, deep vein thrombosis, stroke loci, pulmonary embolism and atherosclerosis.

10 27. Reagent according to any of claims 1-25 for the in vitro analysis of affinity labelled biomolecules, preferably biomolecules labelled with biotin or derivatives thereof, wherein the amount of affinity label bound to the biomolecule is determined.

15 28. Method for diagnosis or treatment of a mammalian condition or disease, wherein a reagent according to any of the previous claims is conjugated to a biomolecule, and wherein said conjugated biomolecule is added to the blood circulation of a mammal and kept therein for a cer-
20 tain time in order to be concentrated to the target tissue or cells on which it is to be detected and/or exert its therapeutic action, wherein the conjugated biomolecules not being attached to the target tissue is completely or partially removed from blood circulation by
25 the administration of a protein specifically binding to the affinity ligand or by passing the mammalian blood or plasma through an affinity column specifically adsorbing the conjugated biomolecule by specific interaction with the affinity ligand.

30 29. Method for diagnosis or treatment of a mammalian condition or disease, wherein a reagent according to any of claims 1-26 provided with a radionuclide is conjugated to a biomolecule, or alternatively, the reagent is

conjugated to the biomolecule prior to attachment of the radionuclide, and the said radioactive conjugated biomolecule is added to the blood circulation of a mammal and kept therein for a certain period of time in order to be concentrated to the target tissue or cells on which it is to be detected and/or exert its therapeutic action, wherein the biomolecules that are not being attached to the target tissue are completely or partially removed from the blood circulation by administration of a protein specifically binding to the affinity ligand or by passing the mammalian blood or plasma through an affinity column specifically adsorbing the conjugated biomolecule by specific interaction with the affinity ligand.

30. Kit for extracorporeally eliminating or at least reducing the concentration of a non-tissue-bound therapeutic or diagnostic biomolecule conjugate, which has been introduced to a mammalian host and kept therein for a certain time in order to be concentrated to the specific tissues or cells by being attached thereto, in the plasma or whole blood of the vertebrate host, said kit comprising a therapeutic or diagnostic biomolecule, a reagent according to any of claims 1-26 for simultaneous conjugation of an affinity ligand and an effector agent to a biomolecule, means for extracorporeal circulation of whole blood or plasma from the vertebrate host, an optional plasma separation device for separation of plasma from blood, an extracorporeal adsorption device, and a means for return of whole blood or plasma without or with low concentration of non-tissue-bound target specific therapeutic or diagnostic agent to the mammalian host, wherein the adsorption device comprises immobilized receptors specific towards an affinity ligand.

31. A kit according to claim 30, wherein the effector agent is chosen from the group consisting of synthetic or naturally occurring toxins, enzymes capable of converting a pro-drug to an active drug,
5 immunosuppressive agents, immunostimulating agents, and radionuclide binding/bonding moieties with or without the radionuclide.

32. A kit according to claims 30 and 31, wherein the affinity ligand is biotin, or a biotin derivative having
10 essentially the same binding function to avidin or streptavidin as biotin, and the immobilized receptor is avidin or streptavidin, or any other derivatives, mutants or fragments of streptavidin having essentially the same binding function to biotin.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01241

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, A61K 39/00, A61K 47/48, A61K 51/00, A61K 49/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9729114 A1 (BOARD OF REGENTS OF THE UNIVERSITY OF WASHINGTON), 14 August 1997 (14.08.97), see e.g. page 38-42, page 24 - page 29, page 14 - page 15 and claims --	1-32
X	WO 9604313 A1 (IMMUNOMEDICS, INC.), 15 February 1996 (15.02.96) --	1-5, 24-27
X	WO 8910140 A1 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED), 2 November 1989 (02.11.89), see example 5 and claims --	1-5, 24-27

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

4 November 1999

Date of mailing of the international search report

08 -12- 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01241

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0310361 A2 (BECKMAN INSTRUMENTS, INC.), 5 April 1989 (05.04.89), see column 22 - column 23 and figures 6-10 --	1
A	US 5310916 A (KENNETH A. JACOBSON ET AL), 10 May 1994 (10.05.94), see column 2 - column 3 --	20-27
A	WO 9302105 A1 (HYBRITECH INCORPORATED), 4 February 1993 (04.02.93), see page 12 - page 13, page 29, last paragraph - page 21, page 33 - page 34 and claims --	1-5,12,24
A	Proc. Natl. Acad. Sci., Volume 93, May 1996, Vladimir R. Muzykantov et al, "Immunotargeting of antioxidant enzymes to the pulmonary endothelium", page 5213 - page 5218, see page 5214 and 5217-5218 --	1,24
A	EP 0618192 A1 (BOEHRINGER MANNHEIM GMBH), 5 October 1994 (05.10.94) --	1,20-24
A	US 5134071 A (ERIC GAETJENS), 28 July 1992 (28.07.92) --	1,20,24
X	Chemical Abstracts, Volume 126, No 20, 19 May 1997 (19.05.97), (Columbus, Ohio, USA), Otusji, Eigo et al, "Decreased renal accumulation of biotinylated chimeric monoclonal antibody-neocarzinostatin conjugate after administration of avidin", THE ABSTRACT No 258425, Cancer Res. 1997, 88 (2), 205-212 --	28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01241

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Immunology, Volume 147, No 1, July 1991, Alison Tutt, "Trispecific F(ab') ₃ derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic t cells", page 60 - page 69, see page 2, left column --	1,3,11,25
X,P	WO 9904820 A2 (PHARMACIA & UPJOHN AB), 4 February 1999 (04.02.99), see claims 16, 17, 27; page 22 - page 25, line 8; page 19 -- -----	1,11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE99/01241

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **28, 29**
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE99/01241

Claims 28, 29 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/09/99

International application No.

PCT/SE 99/01241

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